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Structural characterization of the intra-membrane histidine kinase YbdK from *Bacillus subtilis* in DPC micelles

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ABSTRACT

Bacterial histidine kinases (HKs) play a critical role in signal transduction for cellular adaptation to environmental conditions and stresses. YbdK from *Bacillus subtilis* is a 320-residue intra-membrane sensing HK characterized by a short input domain consisting of two transmembrane helices without an extracytoplasmic domain. While the cytoplasmic domains of HKs have been studied in detail, the intra-membrane sensing domain systems are still uncharacterized due to difficulties in handling the transmembrane domain. Here, we successfully obtained pure recombinant transmembrane domain of YbdK (YbdK-TM) from *E. coli* and analyzed the characteristics of YbdK-TM using nuclear magnetic resonance (NMR) and other biophysical methods. YbdK-TM was found to form homo-dimers in DPC micelles based on cross-linking assays and analytical ultracentrifugation analyses. We estimated the size of the YbdK-TM DPC complex to be 46 kDa using solution state NMR T_1/T_2 relaxation analyses in DPC micelles. These results provide information that will allow functional and structural studies of intra-membrane sensing HKs to begin.

Introduction

Bacterial cells constantly interact with their environment by monitoring osmolarity, ionic strength, and pH, and by detecting the presence, composition, and concentration of nutrients and harmful compounds. They detect and respond to these environmental stimuli via sets of proteins known as two-component systems [1]. The two-component signal transduction system is a ubiquitously distributed regulatory component of bacteria consisting of a membrane-bound histidine kinase (HK) that acts as a sensor to external stimuli and a response regulator that mediates the cellular response by regulating differential gene expression [2]. Most HKs fall into three major groups, including extracellular (or periplasmic) sensing HKs, intra-membrane sensing HKs, and cytoplasmic sensing HKs [3].

The large majority of HKs are homo-dimeric membrane proteins comprised of several domains, beginning with a short N-terminal cytoplasmic portion connected to an extracellular sensing domain via a transmembrane α-helix. A second transmembrane α-helix connects the extracellular domain to the C-terminal cytoplasmic catalytic domain [1]. The extracellular sensing domains are variable in sequence, reflecting the wide range of environmental signals to which HKs respond. In contrast, the cytoplasmic portion typically includes a conserved catalytic core of approximately 250 residues that contains a set of characteristic sequence motifs labeled the H, N, G1, F, and G2 boxes [4]. The autophosphorylation H-box is found in the N-terminal dimerization and histidine phosphotransfer (His KA) domain. The N, G1, F, and G2 boxes are found in the C-terminal catalytic and ATP-binding (CA) domain. Many variations on this prototypical sensor kinase architecture are known, including missing or additional domains and alternative sensing mechanisms, thus reflecting the diversity of molecules sensed by this microorganism [1,5].

Compared with analyzing the cytoplasmic domains of HKs, studying the sensing domains of HKs is significantly more difficult due to the great sequence variability that reflects the range of different input signals sensed. Among the HKs, intra-membrane sensing HKs (IM-HKs) are characterized by their short input domain consisting solely of two putative transmembrane helices. IM-HKs lack an extracytoplasmic domain, indicating involvement in sensory processing at or from within the membrane interface [2,3,6].

YbdK is a 320-residue HK that is found in the Gram positive bacteria *Bacillus subtilis* and has IM-HK domain architecture. This HK

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has been predicted to possess two N-terminal transmembrane domains (TM1 residues 3–25, extracellular loop residue 26–39, TM2 residues 40–62) and a cytoplasmic domain (residues 67–317) [3,7]. The cytoplasmic domain, which forms a functional dimer, consists of a *h*istidine kinase, *a*denyl cyclase, *m*ethyl-accepting chemotaxis protein, *p*hosphatase (HAMP) domain (residue 67–114), an N-terminal dimerization and histidine phosphotransfer (His KA) domain (residue 126–185), and a C-terminal catalytic and ATP-binding (CA) domain (residue 239–317, Fig. S1A). The His KA domain and CA domain perform autophosphorylation of His 138, the ATP-binding site, and activation of YbdJ (the response regulator) by phosphorylation at Asp 52. The sensing domain of YbdK is located in the two transmembrane helices. (Fig. S1B) [8].

The method by which this IM-HK senses external stimuli in the transmembrane helices is of interest. The YbdK cytoplasmic domain harbors only the standard features characteristic of all HKs, lacking any additional domains that would allow signal detection within the cytoplasm. Therefore, it was proposed that YbdK senses its stimulus either directly inside or at the surface of the cytoplasmic membrane. In the present study, we isolated the transmembrane sensing domain of YbdK (YbdK-TM) in detergent micelles from *E. coli* and characterized the secondary structure by circular dichroism (CD) and nuclear magnetic resonance (NMR). Moreover, YbdK-TM was identified as a dimer based on analytical ultracentrifuge (AUC) and cross-linking experiments. As far as we know, these findings provide the first biochemical data on the transmembrane domain of IM-HK.

Materials and methods

Plasmid construction. The gene encoding the transmembrane domain (Leu2–Lys64) of the YbdK histidine kinase(Locus name: BSU02010) was amplified from the genomic DNA of *Bacillus subtilis* by polymerase chain reaction (PCR). *Nde*I and *Xho*I endonuclease sites, an N-terminal linker (GSGSL) and a C-terminal stop codon were introduced using PCR primers (sense primer 5′-CGC *CAT ATG* GGT TCT GGT TCT TTG TTA TTG TTT ACG GCC GTC AT-3′, antisense primer 5′-CGC *CTC GAG TAG* CTA CTT TGA AAA TAA AAA AGC TAA C-3′, with the italicized bases indicating restriction sites at the stop codon and underlined bases indicating the N-terminal linker [GSGSL]). The resulting PCR product was cloned into the *Nde*I and the *Xho*I sites of the pPosKJ expression vector [9], which resulted in expression of the YbdK-TM fused to 6× His-VHb (*vitreoscilla* hemoglobin) with a thrombin protease cleavage site in front of the GSGSL linker at its N-terminus.

Expression. E. coli strain BL21 (DE3) (Novagen) was used for expression of the protein. The cells were transformed with the pPosKJ plasmid harboring the VHb-YbdK-TM sequence. The cells were grown in LB medium supplemented with 100 μg/ml ampicillin in a baffled flask at 37 °C. VHb-YbdK-TM protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6, and the incubation temperature was decreased from 37 °C (used for bacterial growth prior to induction) to 18 °C. After overnight growth, the cells were harvested by centrifugation at 2700*g* for 20 min at 4 °C and stored at −20 °C in 50 mM Tris, pH 8.0.

Purification and solubilization. The harvested cells were resuspended in buffer A (50 mM Tris [pH 8.0], 150 mM NaCl, 20% [v/v] glycerol) and disrupted with a cell disruptor (Cabinet Cell Disrupter, Constant Systems) using the same buffer at 8 °C under 25 kpsi. To collect the plasma membrane, the supernatant containing the membrane fraction was centrifuged at 200,000*g* for 2 h at 4 °C. For solubilization in detergent micelles, the membrane fraction pellet was solubilized in buffer A containing 60 mM lauryldimethylamine-*n*-oxide (LDAO) (w/v) by mild agitation for 1 h at 4 °C. Following centrifugation at 200,000 × g for 1 h at 4 °C, the supernatant containing the VHb-YbdK-TM was incubated for 30 min under gentle stirring with 10 ml of Ni–NTA resin (Qiagen) equilibrated with Buffer A containing 4 mM (w/v) LDAO/4 mM (w/v) dodecyl-phosphocholine (DPC)/0.1 mM (w/v) 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LMPG). The resin with the bound protein was then packed into a gravity-flow column and washed with five bed volumes of Buffer A with 4 mM LDAO/10 mM DPC/ 1 mM LMPG and 30 mM imidazole. The VHb-YbdK-TM was eluted by the addition of Buffer A with 2 mM LDAO/2 mM DPC/0.1 mM LMPG and 300 mM imidazole. To cleave the VHb fusion portion, the purified protein was incubated with thrombin protease at a 10 unit/1 mg sample ratio over two days at room temperature. The reaction mixture was then dialyzed for 20 h at 4 °C in 30 volumes of Buffer A using a dialysis membrane with a molecular weight cutoff of 6–8 kDa (Spectra/Por, Spectrum Labs). After dialysis, the mixture was incubated at 4 °C for 1 h with 10 ml of Ni–NTA resin equilibrated with Buffer A, and the YbdK-TM was separated from the unbound fraction. Finally, YbdK-TM was purified by size exclusion chromatography using a prep-grade HiLoad 16/60 (ID = 16 mm, length = 60 cm) Superdex 75 column (GE Healthcare) with a buffer containing 20 mM sodium acetate (pH 4.8) and 2 mM DPC. Distribution coefficients (K_D) of protein standards and the YbdK-TM detergent complex were determined by $K_D = (Ve - V0)/(Vt - Vo)$ (*Ve*: elution volume, *V0*: void volume, *Vt*: the total excluded volume) [10].

Ribonuclease A (molecular mass: 13,700 Da), chymotrypsinogen (molecular mass: 25,000 Da), ovalbumin (molecular mass: 43,000 Da), and albumin (molecular mass: 67,000 Da) were used to calibrate the column for YbdK-TM.

CD (circular dichroism). CD measurements were performed at room temperature with a Jasco-710 spectropolarimeter (Jasco) using a quartz cell with a path length of 1 mm. Far-UV CD spectra were monitored from 260 to 195 nm using a protein concentration of 31.2 μM in a buffer of 20 mM sodium acetate (pH 4.8) and 20 mM DPC with 50 millidegree sensitivity, a response time of 1 s, and a scan speed of 12 nm/min. Spectra were recorded as an average of five scans. A background CD spectrum acquired from buffer was subtracted from the sample CD data. The composition of the secondary structure elements was analyzed using the neural network-based software CDNN version 2.1 [11].

Cross-linking. A solution containing the YbdK-TM domain (70 μM) in DPC micelles was incubated with 0% and 0.2% (v/v) glutaraldehyde in conjugation buffer (50 mM HEPES, pH 7.7/20 mM DPC) at room temperature for 2 h. After the reaction was quenched with 1 M Tris·Cl (pH 8.0) for 30 min, the cross-linked results were analyzed on an 18% SDS/polyacrylamide gel [12].

NMR spectroscopy. To obtain a ^{15}N-labeled YbdK-TM NMR sample, protein samples were prepared from cells grown in M9 minimal medium supplemented with $^{15}NH_4Cl$. NMR measurements were performed with Avance II 900 spectrometers (Bruker, Rheinstetten, Baden-Württemberg, Germany) equipped with a cryogenic triple resonance probe. All NMR spectra were processed with NMRPipe and analyzed with SPARKY 3.110. The 2D ^{1}H–^{15}N transverse relaxation optimized spectroscopy (TROSY) spectra of 0.4–0.6 mM ^{15}N-labeled YbdK-TM were measured under a variety of conditions with various pHs and detergents. The concentration of DPC was calibrated using 1D ^{1}H NMR spectra with 2,2-dimethyl-2-silapentane-5-sulfonic acid as the reference. The 3D ^{1}H–^{15}N–1H nuclear over hauser enhancement spectroscopy (NOESY)–TROSY experiments were performed with 0.8 mM of uniformly ^{15}N-labeled YbdK-TM in a buffer containing 20 mM sodium acetate, pH 4.8/∼150 mM deuterated DPC/10% D_2O.

^{15}N NMR relaxation. ^{15}N-relaxation experiments for the backbone amide groups were carried out at 40 °C on a Bruker 900 MHz spectrometer. ^{15}N T_1 values were determined from spec-